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A 3′ Enhancer in the *IL-4* Gene Regulates Cytokine Production by Th2 Cells and Mast Cells

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Summary

Differentiation of naive T cells into mature Th2 cells is associated with the appearance of a complex pattern of DNase I hypersensitive (DH) sites within the *IL-4/IL-13* cytokine gene cluster. We show here that targeted deletion of an inducible DH site, V_A, and the adjacent conserved DH site V (CNS-2) selectively compromises *IL-4* gene transcription by differentiated Th2 cells and mast cells. In mast cells, the deletion abrogates *IL-4* mRNA induction, an effect mimicked by deletion of the transcription factor NFAT1 (NFATc2), which binds DH site V_A. In T cells, the deletion impairs a process of response maturation, defined by progressive increases in IL-4 levels as Th2 differentiation proceeds. These results identify an essential enhancer which regulates *IL-4* gene expression in two important cell lineages in vivo.

Introduction

The production of cytokines by the immune system is critical for determining the outcome of an immune response. Of particular importance are the Th2 cytokines IL-4, IL-5, and IL-13, which are crucial for immunity against extracellular pathogens and helminth parasites but also contribute to the pathology of allergy, asthma, and other atopic diseases (Romagnani, 1994; Sher and Coffman, 1992). IL-5 promotes eosinophil differentiation (Wardlaw et al., 1995) while a fundamental function of IL-4 is to drive the differentiation of naive T cells into Th2 cells which are then capable of transcribing the *IL-4*, *IL-5*, and *IL-13* genes (Seder et al., 1992; O'Garra, 1998). IL-4 and IL-13 have multiple roles, among which is the ability to promote B cell class switching to IgE (Brown and Hural, 1997; Zurawski and de Vries, 1994). IgE, bound by high affinity Fcε receptors on the surface of mast cells, basophils, and eosinophils, causes degranulation and production of inflammatory mediators and cytokines when crosslinked by antigen (Galli, 1993). Subsequent production of IL-4 and IL-13 by mast cells locally may prolong Th2 responses.

Mast cells and Th2 cells express both IL-4 and IL-13, despite having developed from the myeloid and lymphoid lineages of the immune system, respectively (Mekori and Metcalfe, 2000; Mosmann and Coffman, 1989). The process of Th2 differentiation has been extensively studied due to the ease with which naive $CD4^+$ T cell precursors can be isolated from mice and differentiated ex vivo (Mosmann et al., 1986). In naive T cells, signaling through the IL-4 receptor in the presence of antigen stimulation leads to activation of the transcription factors STAT6 and GATA-3, which are essential for establishment of the Th2 differentiation program (Kaplan et al., 1996; Takeda et al., 1996; Zheng and Flavell, 1997; Zhang et al., 1999). Another IL-4/STAT6-induced transcription factor, c-Maf, is important for the inducible expression of the *IL-4* gene in already differentiated Th2 cells (Kim et al., 1999). In contrast, early differentiation of murine mast cells occurs in the bone marrow and requires an entirely different set of stimuli, IL-3 and SCF (stem cell factor, the c-Kit ligand) (Galli and Hammel, 1994). Early mast cells leave the bone marrow, enter their destination tissue (e.g. skin, gastrointestinal mucosa, respiratory mucosa), and complete their differentiation under the influence of local environmental factors (Kitamura, 1989). Because of the difficulty in identifying and isolating large numbers of mast cell precursors, it is not yet possible to reconstitute mast cell differentiation ex vivo. However by culturing whole bone marrow with IL-3, it is possible to obtain cells that resemble early mast cells before they enter the tissues (Galli et al., 1982). When injected into mice, these bone marrow-derived mast cells (BMMCs) home to tissues and complete their differentiation (Nakano et al., 1985). BMMCs are capable of producing IL-4 and IL-13 yet do not express the transcription factors vital for IL-4 and IL-13 production in Th2 cells, STAT6, and GATA-3 (Sherman et al., 1999b; Zon et al., 1991). c-Maf is also not required for mast cell transcription of the *IL-4* gene (Sherman et al., 1999a).

Several laboratories have investigated regulation of IL-4, IL-5, and IL-13 production by Th2 cells (reviewed in Glimcher and Murphy, 2000; Murphy et al., 2000; O'Garra and Arai, 2000). These cytokines are all encoded within a cytokine gene cluster that spans ~200 kb and is strongly conserved among mammalian species (Frazer et al., 1997; Loots et al., 2000). Th2 differentiation correlates with development of a complex pattern of DNase I hypersensitive (DH) sites on the *IL-4/IL-13* locus (Agarwal and Rao, 1998; reviewed in Avni and Rao, 2000; Takemoto et al., 1998) (see Figure 1A). Several of the DH sites correspond to conserved regions which show high sequence identity (~80%) when different mammalian species are compared (Loots et al., 2000; Lee et al., 2001). One of these, conserved noncoding sequence (CNS)-1, is located in the intergenic region between *IL-13* and *IL-4* and overlaps two Th2-specific DH sites, HSS1 and HSS2 (Loots et al., 2000; Takemoto et al., 1998). Deletion of CNS-1 in the context of a human YAC transgene or the endogenous mouse locus resulted in significant (2- to 4-fold) decreases in production of IL-4, IL-5, and IL-13, suggesting a global role for this sequence in transcription of these linked cytokine genes

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(Loots et al., 2000; Mohrs et al., 2001). A second conserved noncoding sequence, CNS-2, is contained within DH site V, a Th2-specific DH site located 3′ of the *IL-4* gene that is constitutively present in both resting and stimulated Th2 cells (Agarwal and Rao, 1998). Adjacent to DH site V is an inducible DH site, V_A, which is apparent only in stimulated Th2 cells; it binds the Th2-specific transcription factor GATA-3 and the inducible transcription factor NFAT1 (NFATp, NFATc2) in chromatin immunoprecipitation experiments and functions as an inducible, Th2-specific enhancer in transient reporter assays (Agarwal et al., 2000).

Work on mast cell cytokine transcription has focused mainly on *IL-4* gene expression. Early studies suggested that the transcription factor complexes binding to *cis* elements in the *IL-4* promoter differed from those observed in Th2 cells (Brown and Hural, 1997). A region within the second intron acts as an enhancer of *IL-4* transcription in transfected mast cell lines (Henkel et al., 1992). It has been shown to bind GATA-1, GATA-2, and PU.1, factors specific to mast cells (Henkel and Brown, 1994). There is also evidence that this region helps maintain the demethylated status and accessibility of the *IL-4* locus in mast cells (Hural et al., 2000). Transformed mast cell lines show DNase I hypersensitivity patterns on the *IL-4/IL-13* locus that are similar to those observed in Th2 cells (Agarwal and Rao, 1998). However, deletion of CNS-1 had no effect on cytokine expression by primary mast cells (Mohrs et al., 2001), suggesting that T cells and mast cells might utilize different regulatory regions to modulate *IL-4* and *IL-13* gene expression.

In this study we compare the regulation of *IL-4* and *IL-13* expression in primary mast cells and Th2 cells. We show that these two cell types develop similar but not identical patterns of DNase I hypersensitivity on the *IL-4* gene. Targeted deletion of two adjacent DH sites, the constitutive DH site V and the inducible DH site V_A, results in a profound decrease in *IL-4* expression by mast cells as well as Th2 cells, suggesting that these two cell types utilize similar underlying mechanisms to regulate *IL-4* gene transcription.

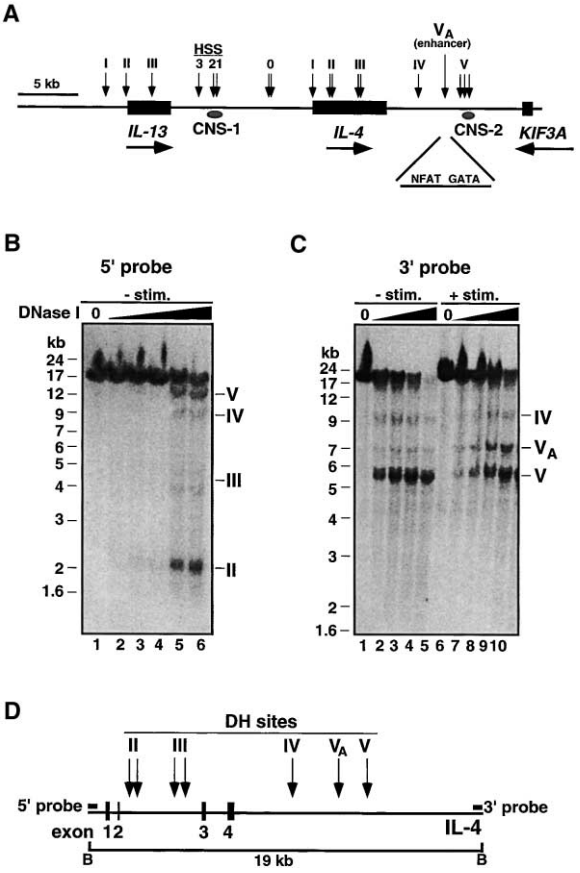

Figure 1. DNase I Hypersensitivity Pattern of the *IL-4* Gene in Mast Cells

(A) Diagram of the *IL-4/IL-13* locus showing the locations of the DNase I hypersensitive clusters (vertical arrows) and the conserved CNS-1 and CNS-2 regions (ovals). The *IL-4* and *IL-13* genes and the last exon of the *KIF3A* gene are shown as black boxes.

(B) DNase I hypersensitivity pattern of unstimulated (− stim.) BMMC using the 5′ *IL-4* probe.

(C) DNase I hypersensitivity pattern of unstimulated (− stim.) and stimulated (+ stim.) BMMC using the 3′ *IL-4* probe.

(D) Diagram of the 19 kb BamHI (B) fragment containing the *IL-4* locus. Exons are shown as black boxes and DNase I hypersensitive sites as vertical arrows. The 5′ and 3′ probes are indicated by black bars.

Results

Similar Patterns of DNase I Hypersensitivity in Mast Cells and Th2 Cells

We first asked whether mast cells and Th2 cells developed the same pattern of DNase I hypersensitivity on the *IL-4* gene. We generated BMMCs from wild-type mice ex vivo by culturing bone marrow precursors with IL-3-containing conditioned medium for 4–6 weeks. The DNase I hypersensitivity pattern of the *IL-4* gene in the differentiated mast cells was almost identical to that observed in Th2 cells (Figure 1B; Agarwal and Rao, 1998), with the exception that site V_A, an inducible DH site in Th2 cells, was weakly apparent in mast cells under resting conditions (Figure 1C, lanes 2–5). Nevertheless, the site still behaved as an inducible DH site, since its intensity increased upon stimulation (Figure 1C, lanes 7–10), and this increase was sensitive to cyclosporin A (CsA) (data not shown). Additional experiments confirmed that mast cells possessed the intergenic DH site, HSS3, observed in the *IL-4/IL-13* locus (Takemoto et al., 1998), as well as DH sites in the *IL-13* gene (data not shown). These results are interesting since BMMC differentiate in response to stimuli different from that to which Th2 cells respond. *IL-4* and *IL-13* expression by BMMC does not require the Th2 transcription factors STAT6 or c-Maf (Sherman et al., 1999a, 1999b; also see Supplemental Figures S1A and S1B at http://www.immunity.com/cgi/content/full/17/1/41/DC1); moreover, BMMC do not express *GATA-3* RNA (Zon et al., 1991) or protein (see Supplemental Figure S1C at http://www.immunity.com/cgi/content/full/17/1/41/DC1).

Deletion of Hypersensitive Sites V and V_A from the Endogenous *IL-4* Gene

In addition to the inducible DH site V_A, the 3′ region of the *IL-4* gene contains two constitutive DH sites, site IV and site V (Figures 1A and 1C). DH site V contains CNS-2

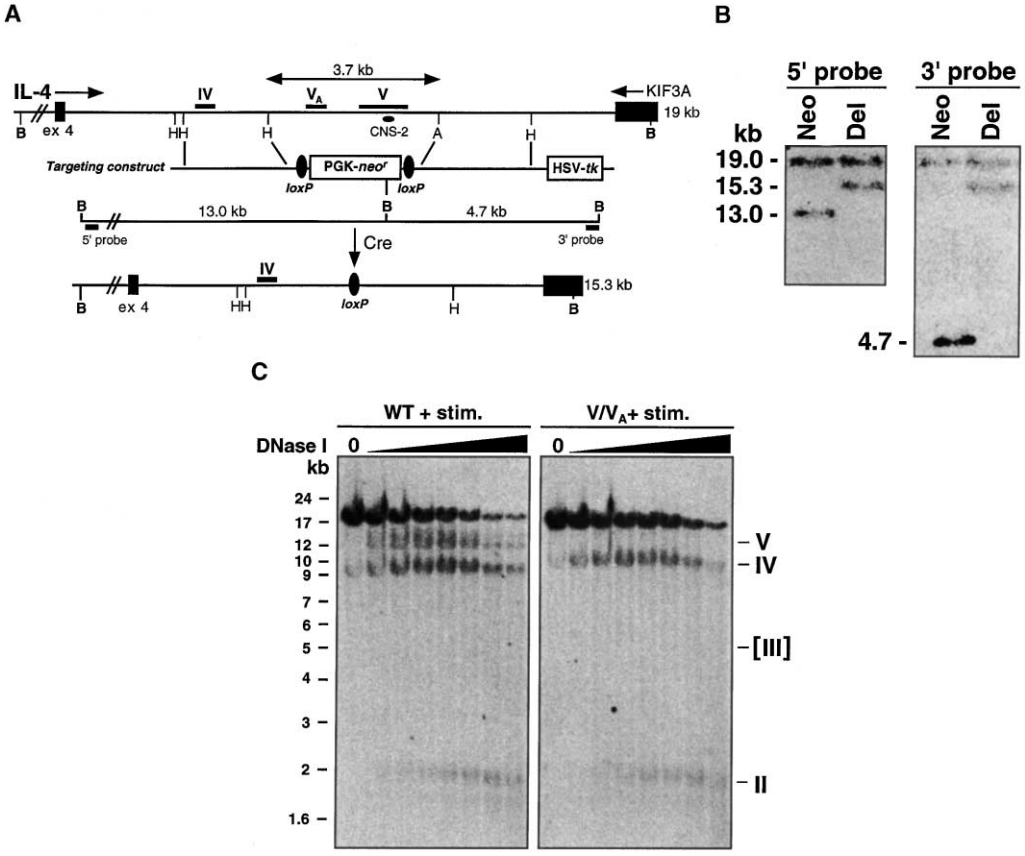

Figure 2. Targeted Deletion of DH Sites V and V_A in the *IL-4* Gene

(A) Diagram of the 3′ region of the *IL-4* locus, the targeting construct used for the site V/V_A deletion, and the *IL-4* locus after Cre-mediated deletion of the PGK-*neo*^{r} selection cassette. Exon (ex) 4 of the *IL-4* gene and the last exon of the *KIF3A* gene are indicated by black boxes. DH sites IV, V, and V_A, the conserved noncoding sequence (CNS-2) present within DH site V, and the 5′ and 3′ probes used for Southern analysis are indicated. Restriction enzyme sites (B, BamHI; H, HindIII; A, AvrII) and *loxP* sites are shown.

(B) Southern analysis of BamHI-digested DNA from targeted ES cells before and after Cre-mediated deletion of the *neo*^{r} cassette. The wild-type *IL-4* locus is contained within a 19 kb BamHI fragment; replacement of the 3.7 kb region including site V and site V_A with the *neo*^{r} cassette (Neo) yielded BamHI fragments of 13.0 and 4.7 kb when screened with the 5′ and 3′ probes, respectively. Cre-mediated deletion of the *neo*^{r} cassette (Del) removed the introduced BamHI site, yielding a BamHI fragment of 15.3 kb with both 5′ and 3′ probes. Only one *IL-4* allele was targeted; hence, the 19kb BamHI fragment from the wild-type allele is observed in all lanes.

(C) Deletion of DH sites V and V_A does not affect formation of other DH sites or induce the appearance of new DH sites in the *IL-4* gene. Wild-type (WT) and V/V_A KO (V/V_A) Th2 cells were differentiated ex vivo from $CD4^+$ T cells. After 3 weeks of differentiation, cells were stimulated with PMA and ionomycin for 4 hr. DNase I hypersensitivity analysis was performed on isolated nuclei using the 5′ probe. Site III, normally a faint DH site, is not visible in this exposure. The 19 kb BamHI parental fragment in the wild-type cells becomes smaller (15.3 kb) when the V/V_A region is deleted and therefore migrates at a slightly faster rate in the gel. Identical results were obtained with wild-type and V/V_A KO mast cells.

while site IV, upstream from sites V and V_A, is not Th2 specific and is seen in both naive T cells and Th1 cells (Agarwal and Rao, 1998). We deleted the adjacent DH sites V and V_A and, separately, DH site IV from the endogenous *IL-4* gene. Here we focus on the effects of deleting DH sites V and V_A; the effects of the site IV deletion will be reported elsewhere. Targeted disruption of DH sites V and V_A was performed by deleting a 3.7 kb fragment containing sites V and V_A from the *IL-4* gene by homologous recombination in ES cells (Figure 2A). The introduced PGK-*neo*^{r} cassette, which had been flanked with *loxP* sites, was then removed by transfection with the Cre recombinase (Figures 2A and 2B). The V/V_A-deleted ES cells were injected into C57BL/6 blastocysts, and mice homozygous for the V/V_A deletion (designated V/V_A KO mice) were generated. $CD4^+$ T cells isolated from 3- to 4-week-old V/V_A KO mice and differentiated under Th2 conditions for three weeks developed a normal DNase I hypersensitivity pattern at other regions of the *IL-4* gene (Figure 2C), as did BMMC differentiated for 8 weeks from the bone marrow of 4-week-old V/V_A KO mice (data not shown). These results indicate that DH sites V and V_A are not necessary to establish the characteristic complex pattern of DNase I hypersensitivity on the *IL-4* gene in either Th2 cells or mast cells.

The V/V_A Deletion Almost Completely Abrogates *IL-4* Expression by Mast Cells

Comparison of wild-type and V/V_A KO mast cells showed that the V/V_A region was critical for *IL-4* production by mast cells. Mast cells from V/V_A KO mice displayed an almost complete loss of the ability to induce *IL-4* mRNA

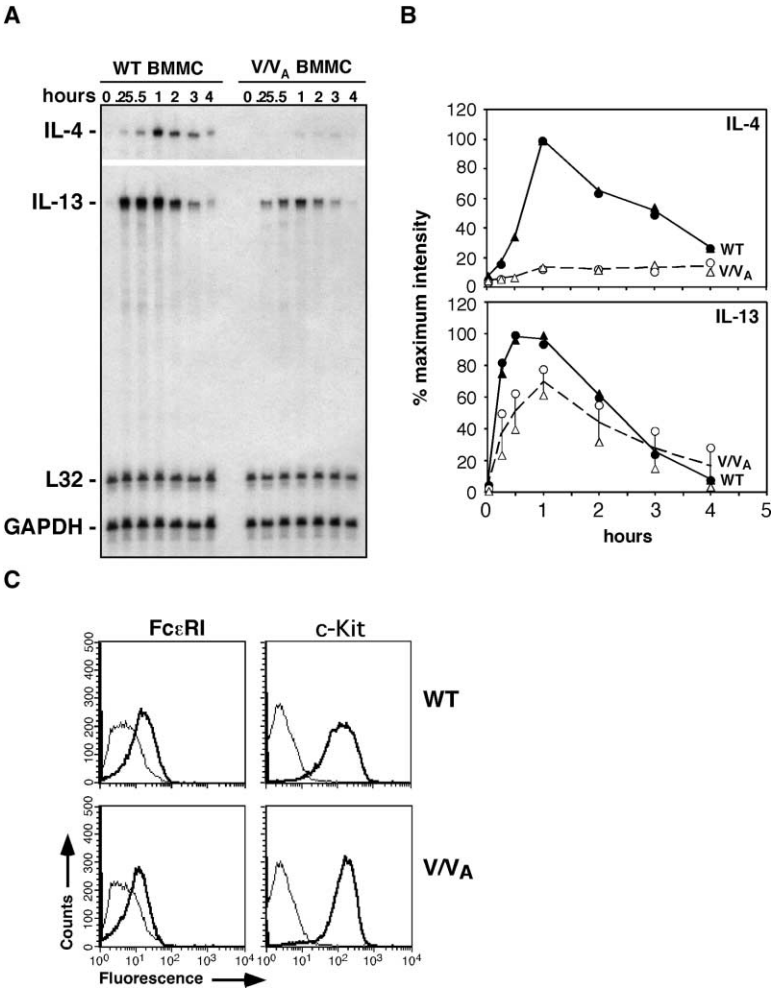

Figure 3. The V/V$_A$ Region Is Essential for *IL-4* Transcription by Mast Cells

(A) RNase protection assay (RPA) to compare cytokine expression by wild-type (WT) and V/V$_A$ KO (V/V$_A$) BMMC. Cells were stimulated over a period of 4 hr. RNA was isolated at the time points indicated and subjected to RPA analysis with the mCK-1b template set. The upper and lower panels were exposed for 5 and 1 hr, respectively.

(B) Quantification of *IL-4* and *IL-13* transcript levels from two independent experiments, indicated by circles and triangles, respectively. Triangles show data from the experiment in (A). Transcript levels at each time point were quantified, and the maximum intensity for each experiment was set at 100%. The other data points were plotted relative to the maximum. The lines show average values at each time point. Solid lines and filled symbols, wild-type BMMC; dashed lines and open symbols, V/V$_A$ KO BMMC.

(C) Equivalent surface expression of c-Kit and FcεRI on wild-type and V/V$_A$ KO BMMC. Cells were stained for FcεRI and c-Kit as described in the Experimental Procedures. Thick lines, FcεRI- or c-Kit-positive staining; thin lines, background controls.

(Figures 3A and 3B). We also observed a decrease of *IL-13* transcript levels, but the magnitude of this effect was lower and more variable between experiments (two independent experiments are represented by circles and triangles in Figure 3B). Decreased cytokine expression in the V/V$_A$ KO mast cells was not due to reduced surface expression of the high affinity IgE receptor, FcεRI, through which the mast cells were stimulated or to a failure of mast cell differentiation as judged by surface expression of c-Kit, the receptor for SCF (Figure 3C). Thus, the major effect of the V/V$_A$ deletion in mast cells was to disrupt *IL-4* gene transcription, with a smaller and more variable effect on transcription of the linked *IL-13* gene.

NFAT1 Deletion Mimics the Effect of the V/V$_A$ Deletion on *IL-4* Expression by Mast Cells

Since the site V$_A$ enhancer is known to bind NFAT1 (Agarwal et al., 2000), we examined cytokine production by mast cells from *NFAT1*-deficient mice (Xanthoudakis et al., 1996). NFAT1$^{-/-}$ mast cells showed no change in the pattern of DNase I hypersensitivity developing on the *IL-4* gene (data not shown), indicating that NFAT1 is not required for remodeling of the *IL-4* locus during BMMC differentiation. However, they showed an almost complete loss of *IL-4* transcript induction, as well as a marked decrease in levels of *IL-13* and *TNF-α* transcripts (Figures 4A–4C). This effect could not be attributed to failure of mast cells to express other NFAT proteins; expression of NFAT2 and NFAT4 was equivalent in wild-type and NFAT1-deficient mast cells (Figure 4D). NFAT1$^{-/-}$ BMMCs and wild-type BMMCs expressed comparable levels of FcεRI and c-Kit (Figure 4E), ruling out defects in development or initial signaling responses to crosslinked IgE. However, the strong dependence of mast cell cytokine production on NFAT1 was unexpected, since Th2 cells lacking NFAT1 show no decrease but rather a mild increase in Th2 cytokine production (Hodge et al., 1996; Kiani et al., 1997; Xanthoudakis et al., 1996).

The V/V$_A$ Deletion Greatly Diminishes *IL-4* Expression by Differentiated Th2 Cells

We evaluated the role of the V/V$_A$ region in Th2 cytokine expression, using CD4$^+$ T cells differentiated under Th2 conditions for 1 to 8 weeks (Figures 5 and 6). For the experiments in Figure 5, Th2 cells were restimulated for different times, after which total RNA was isolated and analyzed by RNase protection assay (RPA). After 1 week of differentiation, V/V$_A$ KO Th2 cells were clearly able to transcribe the *IL-4* gene when restimulated with anti-CD3/anti-CD28 or the stronger stimulus, PMA/iono-

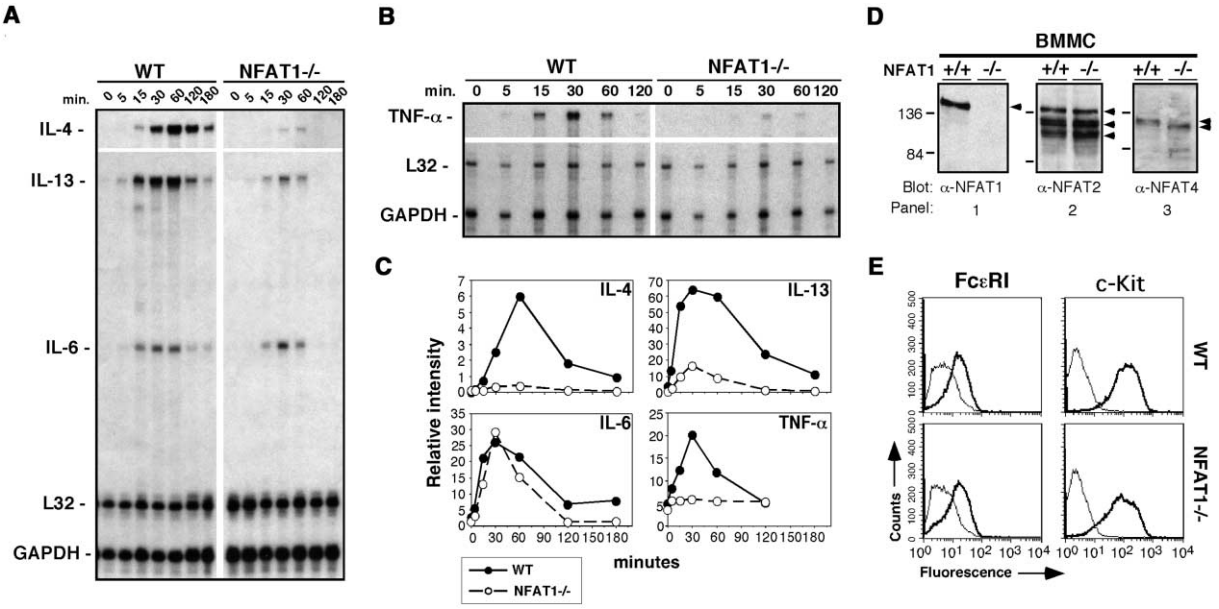

Figure 4. NFAT1-Dependent Transcription of Cytokine Genes in Mast Cells

(A and B) RPA analysis of wild-type (WT) and NFAT1-deficient (NFAT1$^{-/-}$) BMMC stimulated over the course of 3 hr. The mCK-1 (A) and mCK-3 (B) template sets were used. The *IL-4* panels in (A) were exposed for 18 hr while all other panels were exposed for 1 hr.

(C) Quantification of RPA results from (A) and (B). Open symbols, NFAT1$^{-/-}$ BMMC; filled symbols, WT BMMC.

(D) Equivalent expression of NFAT2 and NFAT4 in NFAT1$^{-/-}$ mast cells. Whole-cell extracts were prepared from wild-type and NFAT1-deficient BMMC and pretreated with cyclosporin A to prevent NFAT dephosphorylation during cell lysis. Proteins were separated on a 7% polyacrylamide gel and analyzed by Western blotting to detect NFAT1, NFAT2, and NFAT4 (panels 1, 2, and 3, respectively). Arrowheads mark the positions of NFAT proteins. Minor dephosphorylation of NFAT4 is observed in the NFAT4$^{-/-}$ lysates in panel 3. The specificity of the antibodies was confirmed by Western blot of extracts from HEK cells transfected with recombinant NFAT proteins (data not shown).

(E) Equivalent surface expression of c-Kit and FcεRI on wild-type and NFAT1-deficient BMMC. For details, see legend to Figure 3C.

mycin; however, the levels of *IL-4* transcripts produced were consistently lower than observed for wild-type Th2 cells (Figure 5B, lanes 1–6, and Figure 5A, lanes 1–14). In five independent experiments, V/V$_A$ KO Th2 cells showed 1.3- to 5-fold lower levels of *IL-4* transcripts relative to wild-type Th2 cells after 1 week of Th2 differentiation (mean = 3.3 ± 1.6). The *IL-4* transcription defect was maintained or became more pronounced as Th2 differentiation progressed (Figures 5A and 5B; quantified in graphs at right). The V/V$_A$ deletion also had a detectable but variable effect on transcription of the linked *IL-5* and *IL-13* genes by Th2 cells, with no effect on transcription of the unlinked gene *IL-10* (Figures 5A and 5B; see quantification in accompanying graphs).

The experiments in Figure 5 measure steady-state transcript levels that reflect both synthesis and degradation of cytokine mRNAs. We therefore examined the effect of the V/V$_A$ deletion on cytokine accumulation by ELISA (Figure 6A). This assay confirmed the consistent defect in IL-4 expression by V/V$_A$ KO Th2 cells, as well as the more variable nature of the defect in IL-5 and IL-13 expression. The decrease in IL-4 production was 10- to 12-fold in experiment two and 7- to 17-fold in experiment three (compare black and white bars in Figure 6A). In contrast, levels of IL-5 and IL-13 showed a substantial decrease in experiment two, (IL-5, 4- to 8-fold; IL-13, 3- to 5-fold), but little or no change in experiment three. IL-10 levels were equivalent in wild-type and V/V$_A$ KO Th2 cells in both experiments, a finding that mirrors the results of the RNA analysis in Figure 4.

We also examined IL-4 production at the single-cell level by intracellular cytokine staining of wild-type and V/V$_A$ KO Th2 cells (Figure 6B). Cells from independent cultures (corresponding to Experiments Two and Three of Figure 6A) were stimulated with PMA/ionomycin after 1 or 8 weeks of differentiation under Th2 conditions. In both cases, the fraction of V/V$_A$ KO Th2 cells that stained positive for intracellular IL-4 was approximately 4- to 5-fold lower than the corresponding fraction of IL-4-positive wild-type Th2 cells (Figure 6B). Cells from these cultures were also stained for intracellular IL-4 after stimulation with anti-CD3/anti-CD28; although the fraction of IL-4 positive cells was lower, the 4- to 5-fold difference between wild-type and knockout V/V$_A$ KO Th2 cells was maintained (data not shown). Thus, the site V/V$_A$ region acts as a strong *IL-4* enhancer in Th2 cells and deletion of the region results in a substantial decrease in the amount of IL-4 produced per cell. Since intracellular staining probably detects only the cells expressing the highest levels of IL-4, this decrease is also apparent as a decrease in the frequency of IL-4-producing Th2 cells in the population.

The V/V$_A$ Region Does Not Act as a Boundary Element for the *IL-4* Gene

The V/V$_A$ region is located 3′ of the *IL-4* gene in a location appropriate for a boundary element between the *IL-4* and *KIF3A* genes (Figure 1A). *KIF3A* expression is ubiquitous but occurs predominantly in the central nervous system (Kondo et al., 1994). RT-PCR analysis showed

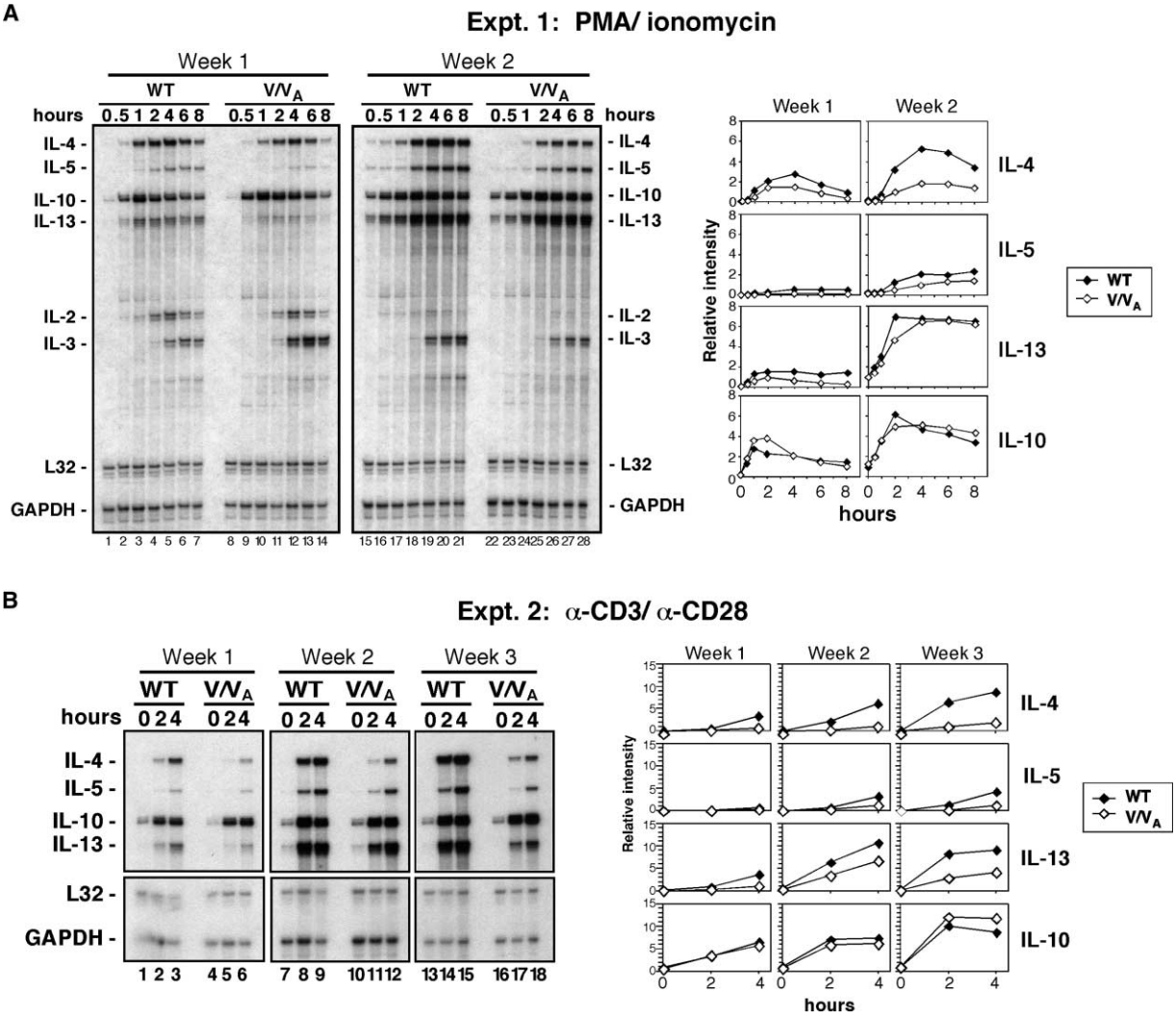

Figure 5. The V/V_A Region Regulates *IL-4* Transcription by Th2 Cells

Wild-type (WT) and V/V_A KO (V/V_A) Th2 cells, differentiated for 1, 2, or 3 weeks ex vivo as indicated, were stimulated over the course of 8 hr (A) or 4 hr (B). Cytokine transcript levels were monitored by RPA using the mCK-1b (A) or mCK-1 (B) templates. Levels of cytokine transcripts were quantified and plotted to the right of each autoradiograph after normalization of the cytokine bands to the L32 standards in each lane. Open symbols, V/V_A KO Th2 cells; filled symbols, wild-type Th2 cells.

(A) Response to PMA/ionomycin stimulation.

(B) Response to anti-CD3/anti-CD28 (α-CD3/α-CD28) stimulation.

that *KIF3A* is expressed in both Th1 and Th2 cells but not in mast cells; the V/V_A deletion neither decreased *KIF3A* expression in Th1 cells and Th2 cells nor induced *KIF3A* expression in mast cells (data not shown).

Discussion

Effects on Mast Cells

We show here that deletion of a region containing a highly conserved noncoding sequence, CNS-2, and an inducible *IL-4* enhancer, DH site V_A, impairs transcription of the *IL-4* gene in both mast cells and Th2 cells. In contrast, deletion of the transcription factor NFAT1, which binds site V_A, has different effects in mast cells and Th2 cells. NFAT1-deficient mast cells show decreased *IL-4* expression (this report), whereas NFAT1$^{-/-}$ Th2 cells show increased expression of *IL-4* mRNA, especially at late time points after stimulation (Hodge et al., 1996; Kiani et al., 1997). In both NFAT1-deficient cell types, there is no change in expression of NFAT2 and NFAT4, and induction of DH site V_A occurs normally (S.A. and D.C.S., unpublished data), implying that other NFAT proteins can bind this enhancer region following stimulation. In T cells, binding of either NFAT1 or NFAT2 leads to productive *IL-4* transcription: IL-4 production is abrogated only when both proteins are absent (Peng et al., 2001), and both constitutively active proteins positively regulate *IL-4* expression in retrovirally infected Th2 cells (S. Monticelli and A.R., unpublished data). However, in mast cells our results suggest that NFAT1 is primarily responsible for *IL-4* gene transcription. A plausible hypothesis to explain these data is that in Th2 cells, NFAT1

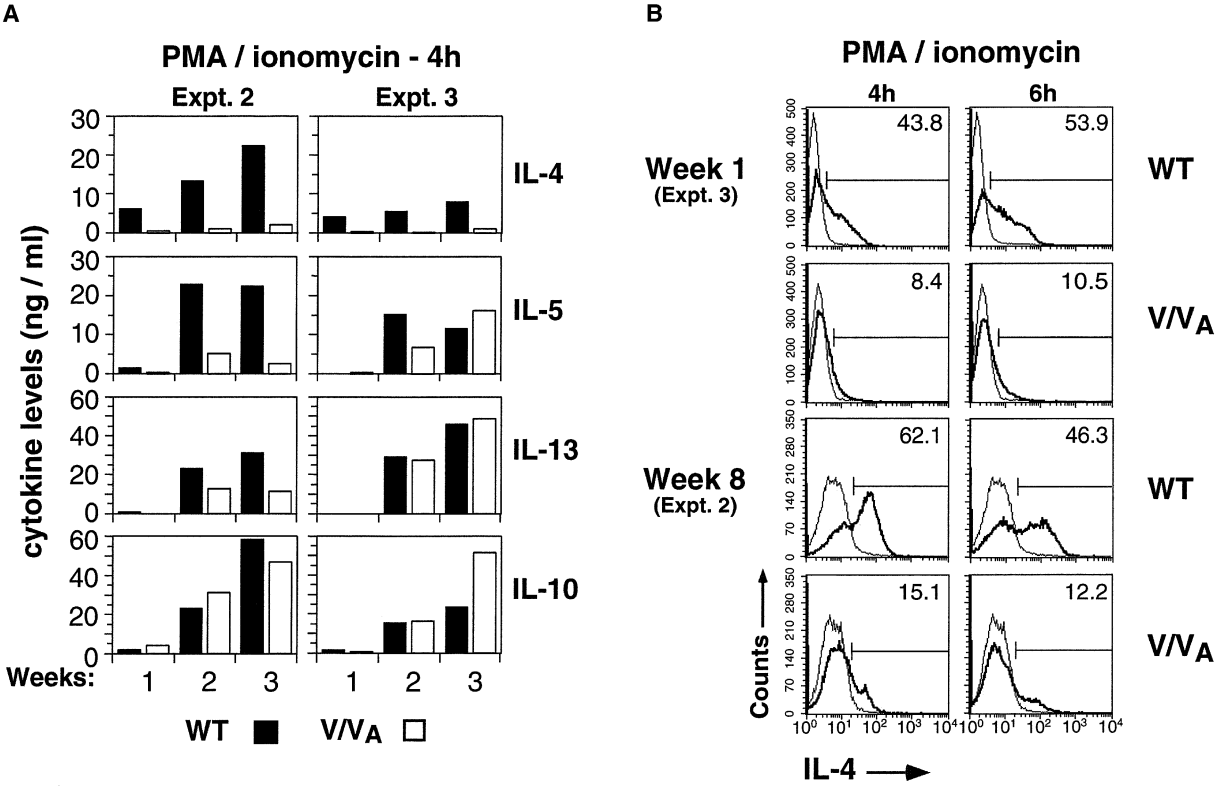

Figure 6. The V/V$_A$ Region Is Essential for IL-4 Protein Production by Th2 Cells

(A) ELISA analysis. Two sets of wild-type (WT) and V/V$_A$ KO (V/V$_A$) CD4$^+$ T cell cultures, designated "Expt. 2" and "Expt. 3," were differentiated over the course of 3 weeks. At the end of each week, each culture was stimulated for 4 hr with PMA and ionomycin, and cell supernatants were collected and assayed for IL-4, IL-5, IL-13, and IL-10 cytokines. Experiment two corresponds to the same culture and stimulation as experiment two of Figure 5B; cells were lysed for RNA immediately after supernatants had been collected. Experiment three is a separate set of cultures. Unstimulated cells produced no detectable cytokines.

(B) Intracellular cytokine staining for IL-4. Wild-type and V/V$_A$ KO CD4$^+$ T cells were differentiated for 1 week (top four panels) or 8 weeks (bottom four panels) under Th2 conditions, then either left unstimulated (thin lines) or treated with PMA and ionomycin (thick lines) for 4 or 6 hr, as indicated. The percentage of cells expressing IL-4 (numbers in upper right corner of each graph) was analyzed by flow cytometry.

and NFAT2 cooperate equivalently with GATA-3 at the site V$_A$ enhancer while in mast cells, NFAT1 interacts preferentially with GATA-1 and GATA-2. Alternatively, NFAT1 and NFAT2 may interact similarly with T cell coactivator proteins but differentially with mast cell-specific coactivators. We are currently testing these hypotheses.

The DNase I hypersensitivity pattern developing on the *IL-4* gene was very similar in primary mast cells and Th2 cells. This similarity was unexpected since these two cell types differentiate in response to different stimuli which utilize unrelated signal transduction pathways; moreover, STAT6 and GATA-3, which are essential for Th2 differentiation, do not play a role in mast cell cytokine production (Sherman et al., 1999b; Zon et al., 1991; see Supplemental Figure S1 at http:www.immunity/com/cgi/content/full/17/1/41/DC1). Mast cell differentiation induced by IL-3 is likely to involve the STAT family member STAT5 (Hural et al., 2000), which has a DNA binding specificity similar to that of STAT6 and other STAT family proteins (Xu et al., 1996); similarly, although mast cells do not express GATA-3, they express GATA-1 and GATA-2 (Martin et al., 1990; Zon et al., 1991) which bind to DNA with similar selectivity as other GATA proteins (Weiss and Orkin, 1995). It therefore seems likely, as previous work suggests (Weiss and Brown, 2001), that the similar DNase I hypersensitivity patterns in Th2 cells and mast cells reflect the participation of related STAT and GATA transcription factors in these two cell types.

Effects on Th2 Cells

We have shown that the *IL-4* expression defect of the V/V$_A$ KO Th2 cells becomes more apparent as Th2 differentiation progresses. As depicted in Figure 5, wild-type Th2 cells show increased induction of *IL-4*, *IL-5*, *IL-13*, and *IL-10* cytokine mRNAs with increasing times of Th2 differentiation. This phenomenon, which we provisionally refer to as "Th2 maturation," likely reflects both an increased number of cytokine-expressing cells in the population as well as an increased level of cytokine expression per cell (Figure 6B). While we observed some variation in the extent to which IL-4 expression was impaired in V/V$_A$ KO Th2 cells at week 1, there was always a striking difference at later weeks (Figures 5 and 6). Thus, the process of Th2 maturation is impaired in site V/V$_A$ KO Th2 cells, in that even cells differentiated

for 8 weeks do not express the levels of cytokine observed in wild-type Th2 cells (Figure 6B).

The site V/CNS-2 region is unusual in that it is demethylated in naive T cells prior to antigen encounter; almost all other tested regions of the *IL-4/IL-13* locus are strongly methylated in naive T cells and become gradually demethylated during the course of Th2 differentiation in a manner that correlates with high-level IL-4 expression (Lee et al., 2002). This might suggest that CNS-2 maintains the *IL-4* locus of naive T cells in a poised state, ready for the widespread changes in chromatin structure that accompany Th2 differentiation (Avni and Rao, 2000). However, this scenario is ruled out by our finding that the V/V_A deletion does not interfere with formation of the other DH sites in the *IL-4* gene. Instead, the deletion seems to interfere with high level IL-4 expression as described above, suggesting that the V/V_A region might orchestrate demethylation in other regions of the *IL-4* gene. We are currently testing this possibility.

Overall Aspects

It is striking that the V/V_A deletion, which includes CNS-2, affects *IL-4* transcription in both mast cells and Th2 cells. Previous work had suggested that these two cell types use different regulatory elements to control *IL-4* transcription. An intronic enhancer was shown to bind mast cell-specific transcription factors and regulate the accessibility and methylation status of the *IL-4* gene in mast cells (Henkel et al., 1992). Conversely, CNS-1, a highly conserved sequence in the intergenic region between the *IL-4* and *IL-13* loci (Loots et al., 2000; Mohrs et al., 2001) was shown to function selectively in Th2 cells, since its deletion had no effect on IL-4 expression by mast cells. This scenario, in which regulation of a single gene in two different cell types occurs through utilization of different *cis* elements, has also been observed for the CD8α/β locus; in this case, different DH clusters control CD8 expression in mature T cells and intraepithelial lymphocytes (IELs) (Hostert et al., 1997; Ellmeier et al., 1998). Our results highlight an alternate mechanism to modulate gene expression, in which the same regulatory elements bind distinct but related transcription factors in different cell types (Weiss and Brown, 2001).

The V/V_A deletion also has variable, less striking effects on expression of the linked genes *IL-5* and *IL-13*. These results may be compared with previous work in which deletion of CNS-1 caused a global decrease in expression of IL-4, IL-5, and IL-13 by Th2 cells but had little effect on cytokine production by mast cells (Mohrs et al., 2001). Why might the CNS-1 and V/V_A deletions have different effects on cytokine production by Th2 cells and mast cells? The V/V_A deletion is more complex since it eliminates two potentially important elements, the V_A enhancer and DH site V, which contains CNS-2. Analogous to CNS-1, CNS-2 may in fact be a global regulator of cytokine production in Th2 cells and mast cells, but deleting it in conjunction with the site V_A enhancer may focus the effects of the combined deletion on *IL-4* expression, thereby rendering the effects on *IL-5* and *IL-13* less prominent. This hypothesis may be tested by examining the individual effects of site V_A and CNS-2 deletions and by combining the CNS-2 deletion with deletion of CNS-1. If CNS-1 and CNS-2 indeed have redundant functions, a combined CNS-1/CNS-2 deletion might have major effects on transcription of the entire *IL-4/IL-5/IL-13* cytokine cluster in mast cells as well as Th2 cells.

Experimental Procedures

Animals

All mice were maintained in pathogen-free conditions in barrier facilities at the Center for Animal Resources and Comparative Medicine at Harvard Medical School. NFAT1$^{-/-}$, STAT6$^{-/-}$, and c-Maf$^{-/-}$ mice have been described previously (Xanthoudakis et al., 1996; Kaplan et al., 1996; Kim et al., 1999). STAT6$^{-/-}$ mice were a gift from Dr. Michael Grusby, and c-Maf$^{-/-}$ mice were a gift from Dr. Laurie Glimcher. NFAT1$^{-/-}$ mice on a mixed C57BL/6-129 background were backcrossed to C57BL/6 for five generations, and C57BL/6 mice were used as wild-type controls. BMMC from BALB/c mice was used as controls for both the STAT6$^{-/-}$ and c-Maf$^{-/-}$ experiments.

BMMC Generation and Stimulation

Mice were sacrificed at 8–12 weeks of age, and bone marrow was flushed from the femurs and tibias of each mouse with Razin medium (RPMI 1640-BioWhittaker, supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, nonessential amino acids, and 5 mM HEPES). Cells were resuspended at approximately 4–5 $\times$ 10^5 cells/ml in 50% Razin medium:50% WEHI-3 (ATCC) conditioned medium as a source of IL-3. Cells were cultured in 50% WEHI-3 media for at least 4 weeks before analysis, at which time the percentage of mast cells in the culture was >99% as assessed by staining with toluidine blue.

For stimulation, BMMC were resuspended in Razin medium at 10×10^6 cells/ml. Cells were sensitized in 3 μg/ml anti-dinitrophenol (DNP) rat IgE (Zymed) for 1 hr at 37°C. After removal of the cells from IgE-containing medium, FcεRI-bound IgE was then crosslinked for the indicated times with mouse anti-rat IgG F(ab)$'_2$ fragments (Jackson Immunoresearch Laboratories) at 25 μg/ml.

DNase I Hypersensitivity

DNase I hypersensitivity of the *IL-4* locus was analyzed as previously described (Agarwal and Rao, 1998). Southern blotting of BamHI-digested DNA was performed using the 5′ and 3′ *IL-4* probes (Agarwal and Rao, 1998).

Gene Targeting and Generation of V/V_A KO Mice

The targeting construct was created in pLNTK (Gorman et al., 1996), which contains a PGK-*neo*^{r} cassette for positive selection and an HSV-*tk* gene for negative selection of random integration events. The *neo*^{r} cassette is flanked by *loxp* sites, enabling Cre-mediated deletion of the introduced selection cassette. The construct contained as its 5′ arm a 1.8 kb HindIII/HindIII fragment and as its 3′ arm a 1.8 kb AvrII/HindIII fragment, for deletion of 3.7 kb encompassing DH sites V and V_A. The construct was electroporated into TC1 ES cells, and cells were selected with G418 and gancyclovir. For Cre deletion, targeted ES cells were electroporated with the pMC-CreN expression vector, and individual colonies were screened by Southern analysis (Agarwal and Rao, 1998) for successful and complete deletion. Positive colonies were subcloned, expanded, and injected into C57BL/6 blastocysts for germline transmission. Mice were bred to homozygosity on the C57BL/6 background.

T Cell Differentiation and Stimulation

Spleen and lymph nodes were isolated from V/V_A KO and C57BL/6J mice (3–4 weeks old). CD4$^+$ T cells were purified using magnetic beads (Dynal) according to the manufacturer's instructions. Th2 cells were differentiated ex vivo as previously described (Agarwal et al., 2000; Agarwal and Rao, 1998).

For stimulation, differentiated Th2 cells ($10–20 \times 10^6$) were resuspended in cytokine- and antibody-free medium at a concentration of 1×10^6/ml and stimulated for the indicated times with 20 nM phorbol myristate acetate (PMA) and 2 μM ionomycin as previously described (Agarwal et al., 2000; Agarwal and Rao, 1998). Where plate-bound anti-CD3/anti-CD28 was used to stimulate Th2 cells, plates were coated for 2 hr at 37°C with 0.3 mg/ml goat anti-hamster

antibody (Sigma) in PBS. Plates were then rinsed one time with PBS before the addition of cells. Cells were washed in PBS and resuspended in media containing 1 μg/ml each of anti-CD3ε and anti-CD28 (BD Pharmingen) and added to the coated flasks for the indicated times.

RNase Protection Assays (RPA)

Total RNA was isolated from cells using Ultraspec (Biotecx). RPA analysis was performed on 2–5 μg of total RNA per time point using the Riboquant Kit (BD Pharmingen) according to the manufacturer's instructions. The mCK-1 and mCK-1b template sets are identical except for the inclusion of an *IL-6* probe in mCK-1 and an *IL-3* probe in mCK-1b. The mCK-3 template set contains a probe for *TNF*-α mRNA. Bands in RPAs that corresponded to cytokine transcripts were quantified through use of the Phosphorimager and Image Quant program (Molecular Dynamics). Cytokine mRNA bands were normalized to the corresponding *L32* mRNA standard bands in each lane, and relative intensity values were plotted.

FcεRI and c-Kit Staining of BMMCs

Approximately 0.5–2 × 10^6 WT, V/V_A KO, and NFAT1$^{-/-}$ BMMCs were washed twice with PBS/1% fetal bovine serum (FBS) and then incubated at 4°C with 5 μg/ml anti-CD16/anti-CD32 (2.4G2, BD Pharmingen) in PBS to block binding of antibodies to the FcγIII/II receptors (Fc block). All remaining steps were carried out at 4°C. Mouse anti-DNP IgE (Sigma) was then added to the cells at 20 μg/ml to saturate the FcεRI for 50 min. During the last 25 min of the incubation, biotinylated anti-c-Kit antibody (BD Pharmingen) at 15 μg/ml was added (none was added for background control). Cells were washed twice with PBS/1% FBS and resuspended in Fc block. Ten μg/ml of anti-IgE-FITC (fluorescein isothiocyanate) or 10 μg/ml of anti-IgG1-FITC as a background control and 2 μg/ml streptavidin-PE (phycoerythrin) (BD Pharmingen) were added to the cells which were incubated in the dark for 30 min. Cells were then washed twice with PBS/1% FBS and resuspended in PBS, and 50,000 events were analyzed by flow cytometry using the FacsCalibur (Becton Dickinson) and Cell Quest software.

Antisera and Western Blotting

For anti-NFAT Western blots, wild-type and NFAT1-deficient BMMC were pretreated with 1 mM cyclosporin A for 30 min to prevent dephosphorylation of NFAT or other phosphoproteins during cell lysis. Whole-cell extracts were prepared from Th2 cells and BMMC by resuspending cells in 2× RSB (40 mM Tris-HCl [pH 7.8], 10 mM EDTA, 60 mM sodium pyrophosphate, 0.2 μg/ml aprotinin, 50 μM leupeptin, and 0.4 mM PMSF). An equal volume of 10% SDS was added, and the samples were boiled. Lysates were run on polyacrylamide gels as indicated in the figure legends and transferred to nitrocellulose. Lysates of HEK293 cells transfected with NFAT expression constructs were a gift from Dr. Jose Aramburu. Antibodies used in Western blotting were as follows: anti-GATA-3, HG3-31 (Santa Cruz Biotechnology); anti-NFAT1, anti-67.1 (Ho et al., 1994; Shaw et al., 1995); anti-NFAT2, 7A6 (Affinity Bioreagents). Anti-NFAT4 (Lyakh et al., 1997) was kindly provided by Dr. Nancy Rice.

ELISA Analysis of Cytokine Production

Th2 cells were stimulated as described above. At the indicated times, supernatants were collected from the cells and serially diluted to obtain results within the linear range of the assay. Capture and detection antibodies were used at the following concentrations: anti-IL-4 (BD Pharmingen), 1 μg/ml; anti-IL-5 (BD Pharmingen), 2 μg/ml; anti-IL-10 (R&D Systems), 2 μg/ml capture, 400 ng/ml detection; anti-IL-13 (R&D Systems), 3 μg/ml capture, 200 ng/ml detection.

Intracellular Cytokine Staining

Th2 cells were stimulated as described above. During the last 2 hr of stimulation, 10 μg/ml brefeldin A (Sigma) was added to the cells. Cells were collected and washed one time in PBS/1% BSA and one time with PBS alone, after which they were resuspended in 500 μl 4% paraformaldehyde in PBS and fixed at room temperature for 10 min. Fixing was followed by one wash in PBS/1% BSA. After washing, cells were resuspended in 0.5% saponin in PBS/1% BSA and permeabilized at room temperature for 10 min. Cells were then pelleted and resuspended in Fc block at 5 μg/ml in saponin buffer for 5 min at room temperature to block binding of antibodies to the FcγIII/II receptors. Anti-IL-4-PE staining antibody (BD Pharmingen) was then added at 5 μg/ml in saponin buffer and incubated at room temperature in the dark for 20 min. Cells were washed twice with saponin buffer, washed twice with PBS/1% BSA, resuspended in PBS, and placed on ice until analysis. Flow cytometry analysis was performed on 50,000 events per sample on the FacsCalibur (Becton Dickinson) using Cell Quest software.

RT-PCR Analysis of *KIF3A* Expression

RT-PCR on RNA isolated from Th1 cells, Th2 cells, and BMMC was performed for *KIF3A* expression with the Superscript One-Step Kit (Life Technologies) according to the manufacturer's instructions using the following primers: *KIF3A* forward, 5′-TTTGCATATGGACA GACTGGG-3′; *KIF3A* reverse, 5′-GACCCAGCCAGATCCACAA-3′.

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